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PRINCIPAL INVESTIGATOR: Alfred Heller, M.D., Ph.D.
Martin Gross, M.D., Ph.D.
Lisa Won, Ph.D.

CONTRACTING ORGANIZATION: Chicago University
Chicago, Illinois 60637

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6. AUTHOR(S)
Alfred Heller, M.D., Ph.D.
Martin Gross, M.D., Ph.D.
Lisa Won, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Chicago University
Chicago, Illinois 60637

E-Mail: effe@midway.uchicago.edu

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13. ABSTRACT (Maximum 200 Words)

A lysate of an immortalized monoclonal cell line derived from the striatum (X61) contains an activity that is capable of increasing the dopamine content of an immortalized, dopaminergic mouse mesencephalic cell line (MN9D). The activity can be extracted from the cell lysate by isoamyl alcohol/chloroform. NMR spectroscopic analysis demonstrated that a purified fraction from the isoamyl alcohol/chloroform soluble dopaminergic stimulatory activity is a mixture of 80-90% cis-9-octadecenoic acid (oleic acid) and 10-20% cis-11-octadecenoic acid (cis-vaccenic acid). MN9D dopamine increases linearly in the presence of oleic acid over a 48 hour period suggesting the induction of an increased dopaminergic phenotype in these dividing cells. The ability to increase MN9D dopamine by oleic and cis-vaccenic acids is shared by a number of other long-chain fatty acids including arachidonic, linoleic, linolenic, palmitoleic, and cis-13-octadecenoic acid. Studies on the mechanism of elevation of MN9D dopamine by oleic acid suggest that this fatty acid increases both the synthesis and uptake and/or storage of dopamine. The possibility that oleic or other relatively innocuous fatty acids might affect dopaminergic function in primary neurons is intriguing with respect to possible therapeutic approaches to the treatment of dopaminergic cell loss and the motor sequelae of Parkinson's disease.

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INTRODUCTION

This report covers the research conducted under Award Number DAMD17-01-1-0819 during the period September 15, 2003 to September 14, 2004. The prevalence, morbidity and mortality associated with Parkinson's disease (PD) has stimulated an intense investigation of a variety of strategies for the treatment of this disorder (for a detailed review see [1]). PD is a progressive debilitating disorder with a variety of motor dysfunctions including rigidity, bradykinesia and tremor. It is a common affliction affecting some 1.0% of individuals over the age of 55. Approximately one million individuals in North America suffer from PD and its incidence is second only to Alzheimer's disease in terms of neurological disorders. PD is associated with a marked loss in dopamine (DA) [3] secondary to degeneration of the nigrostriatal DA projection. One of the approaches to the treatment of PD in humans and in animal models is the application of neurotrophic agents. Such substances are present in the nigrostriatal projection and support the normal survival and differentiation of mesencephalic DA neurons (for review see Beck [2] and Hefti [4] and references therein). Some 14 such "trophic agents" have been identified and a variety tested in animal models of the disease. Glial derived neurotrophic factor (GDNF) has been reported to enhance the survival of human fetal DA neurons and implants of fetal cells [8, 9] and was shown to be effective in primate models of the disease [7]. Clinical trials of this trophic agent in PD patients were, in fact, initiated [1], but studies by the Amgen pharmaceutical company were recently halted. This development while disappointing, only points to the need to identify additional trophic agents. In this regard, we have, during the period of Army support under Award Number DAMD17-01-1-0819, succeeded in purifying two chemically distinct dopaminergic stimulatory factors from the lysate of the X61 cell, an immortalized hybrid monoclonal cell derived from the corpus striatum. One of factors is most likely a small, highly hydrophilic polypeptide whose specific structure is still under investigation. Recent work has provided a highly purified fraction of this small peptide which should be amenable to structural analysis by mass spectrographic and NMR analysis. The second, in contrast, could be extracted by organic solvents from a concentrate of the X61 lysate and was conclusively demonstrated to include primarily the long-chain, unsaturated fatty acid cis-oleic acid (90%) and cis-vaccenic acid (10%) and has lead to an examination of a series of active fatty acids with one to four unsaturated bonds (Heller et al., [6]). Studies are being conducted on the mechanism of action and potential utility of the small, hydrophilic peptide(s) and long-chain fatty acids as potential pharmacological approaches to the investigation of the Parkinson's disease and its treatment.

BODY

The primary objective of the research supported by Grant DAMD17-01-1-0819 continues to be the purification, identification and mechanism of action of dopaminergic stimulatory factors obtained from the immortalized monoclonal hybrid cell, X61, derived from the corpus striatum [5, 10].

During the period of this report (September 15, 2003 to September 14, 2004), research has been conducted on three specific tasks outlined in the current approved Statement of Work. These include: 1) further purification of a dopaminergic stimulatory factor which would appear to be a highly hydrophilic peptide in the range of 0.4 to 0.8 kDa; 2) purification and identification of an

isoamyl alcohol/chloroform soluble dopaminergic stimulatory factor present in a lysate of the X61 cell; and 3) examination of the mechanism by which these factors elevate cellular dopamine.

Further purification of a small, hydrophilic dopaminergic stimulatory factor obtained from X61 lysate

We have previously described in the detail (see annual reports of October 2002 and October 2003) the isolation and 50-70,000-fold purification of highly hydrophilic, small peptide in the range of 0.4 to 0.8 kDa capable of increasing cellular dopamine. The dopaminergic stimulatory peptide(s) was purified from a low molecular weight fraction of 2 to 3 day autodigested X61 cell lysate which passed through an Amicon YM-5 (MWCO 5 kDa) filter membrane, but was concentrated (i.e., retained by the filter) by a YC-05 membrane (MWCO 0.5 kDa). The hydrophilic nature of this factor, however, appeared to preclude its further isolation by standard reverse phase chromatography. Examination of that fraction of the stimulatory activity, which passes through the YC-05 membrane when the 2 and 3 day, YM-5 ultrafiltrates are pressure concentrated, may prove more amenable to purification. When the solubility of the YC-05 concentrate and ultrafiltrate were tested in a variety of organic solvents, it was found that the latter was much more soluble in methanol than the former. This suggested that the YC-05 ultrafiltrate activity may be less hydrophilic and more likely to interact with the reverse phase chromatography column resin, that is dependent upon hydrophobic interaction. YC-05 ultrafiltrate activity was then purified through the same initial steps as used for the YC-05 concentrate activity including charcoal extraction (which does not bind the activity), concentration by lyophilization, gel-filtration chromatography on a Superdex-peptide column, re-extraction with charcoal, and re-concentration by lyophilization. The product is about one-half as pure (one-half the activity per absorbance at 225 nm) as our purest YC-05 concentrate activity. However, unlike the YC-05 concentrate, the YC-05 ultrafiltrate activity interacts with the resin of a 5 micron, C18 reverse phase column and can be purified by isocratic chromatography in 0.05 % trifluoroacetic acid followed by repeated re-chromatography of the activity, leading to a progressively purer product.

This procedure should generate a single purified species or, at the very least, a preparation with very few, highly enriched molecules (based on mass spectroscopic analysis). In either case, the structure (or structures) can be analyzed by tandem mass spectroscopic separation and then fragmentation analysis, using precise molecular weight measurements to deduce the chemical components (and sequence) of the stimulatory activity. The mass fragmentation analysis will be performed by Alex Schilling in the Proteomics Core Laboratory at The University of Chicago. In addition, the purified product will be subjected to amino acid microsequence analysis and to a sensitive amino acid composition analysis by Giri Reddy in the Polypeptide Core Laboratory at The University of Chicago. Finally, the purified product will be subjected to NMR analysis (dissolved in D_2O) by Josh Kurutz in the Biomolecular NMR Facility at The University of Chicago. The deduced structure or structures will be synthesized and tested to verify or determine the precise identity of the activity.

Purification and identification of an isoamyl alcohol/chloroform soluble dopaminergic stimulatory factor from X61 lysate

The results described below are in press in Neuroscience Letters (see Heller et al. in the Appendix).

Since approximately two thirds of the stimulatory activity present in the lysate does not pass through a 5 kDa filter membrane, we re-examined this "X61 concentrate" by extraction initially with 2M NaCl followed by a 1:1 mixture of isoamyl alcohol/chloroform. Both the 2M NaCl soluble and the organic soluble activities were purified on reverse phase chromatography.

The details of this purification are given below.

The isoamyl alcohol/chloroform soluble activity, as seen in Figure 1, produces a maximal 4.5-fold increase in MN9D dopamine. It is of interest that this activity is not extractable from fresh cell lysate, but, as is the case with the small peptide activity, requires cell digestion with time for the activity to become liberated from some cell component in order to be available for extraction.

The isoamyl alcohol/chloroform soluble stimulatory activity is taken up by a C18 reverse phase column from 70% acetonitrile. The activity can be eluted from this column by an acetonitrile-trifluoroacetic acid gradient (Figure 2). All of the fractions containing activity (31-35) showed absorbance at 215 nm. The vast bulk of absorbance was, however, present in fractions devoid of such activity, suggesting that the reverse phase separation resulted in active fractions containing purified material.

Figure 1

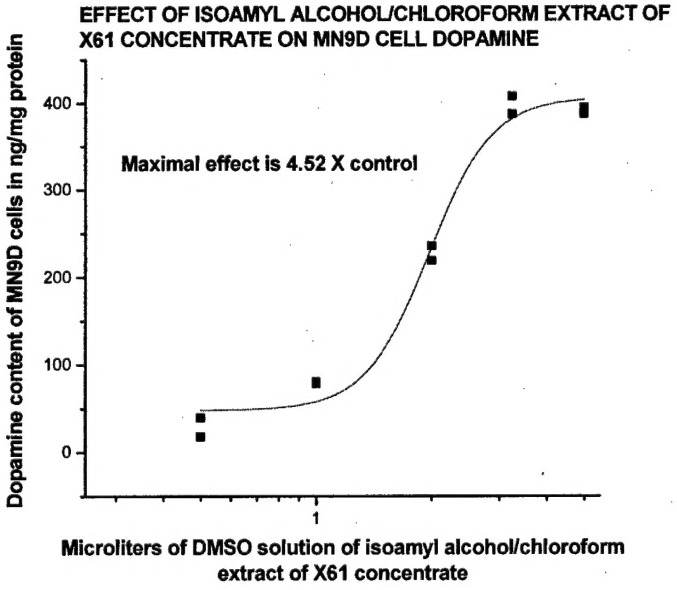
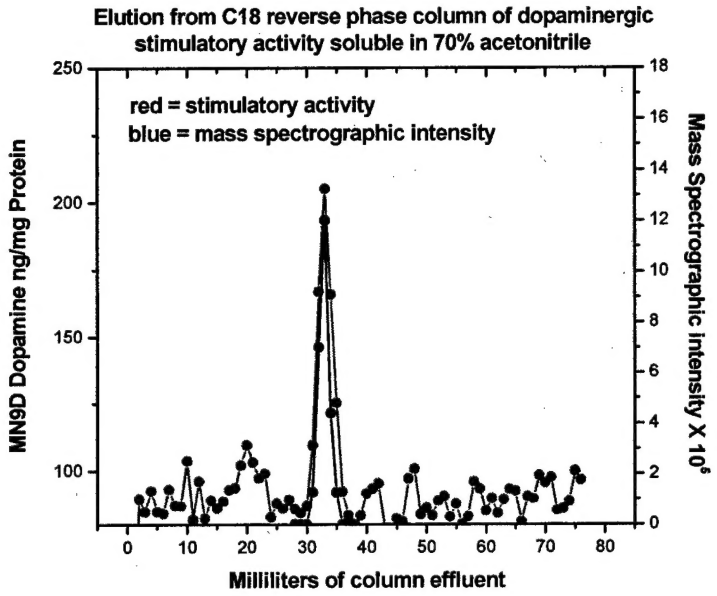


Figure 2



The material contained in this elution peak was then subjected to further purification using a Phenogel gel filtration sizing column (GFC). The dopaminergic stimulatory activity eluted from the Phenogel column in 100% acetonitrile within a single absorbance peak corresponding to GFC fractions #48-51.

NMR spectroscopy, by Dr. Josh Kurutz (Technical Director of the University of Chicago's Biomolecular NMR Facility), demonstrated that this single elution peak from the Phenogel column contained two chemical moieties, the greater of which constituted 90% of the material (Figure 3). The 600 MHz ^{1}H 1D NMR spectrum of the sample showed one major set of peaks and no significant minor peaks, indicating a sample purity of >95%. The ^{13}C 1D NMR spectrum, however, showed two sets of peaks, indicating that the sample contains two species so closely related that they give rise to virtually identical ^{1}H NMR spectra. The minor component made up approximately 10-20% of the total sample. The positions of the ^{1}H and ^{13}C peaks in their spectra definitively rules out the possibility that the sample comprises protein, peptide, DNA, RNA, carbohydrate, glycolipid, steroid, or other cholesterol-related molecules. The positions are instead consistent with those expected from a fatty acid. Comparison of the major species' peaks with ^{1}H and ^{13}C NMR spectra of various fatty acids available on the Sigma-Aldrich company's website showed that they were very similar to those of oleic acid. It remained uncertain whether the unsaturated bond was cis or trans. Pure oleic acid (cis-9-octadecenoic acid) and elaidic acid (trans-9-octadecenoic acid) were obtained from Aldrich, and their ^{1}H and ^{13}C NMR spectra were compared to those of the sample. We found that the major species' peaks were essentially identical to those of oleic acid. This fact, coupled with the mass spectrometry data on the sample, strongly supports the conclusion that the major peak in the sample is oleic acid.

Figure 3

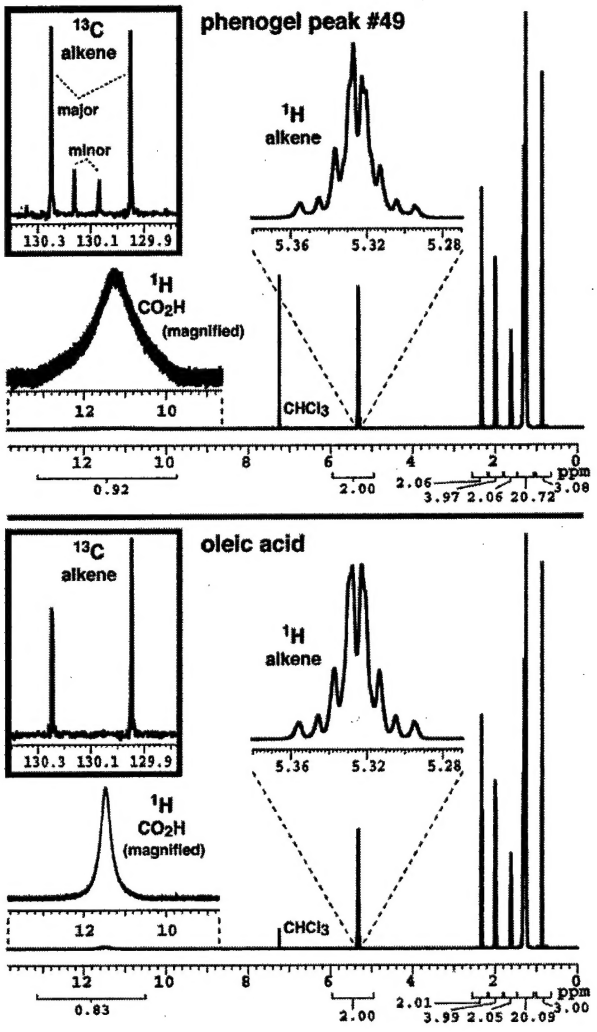
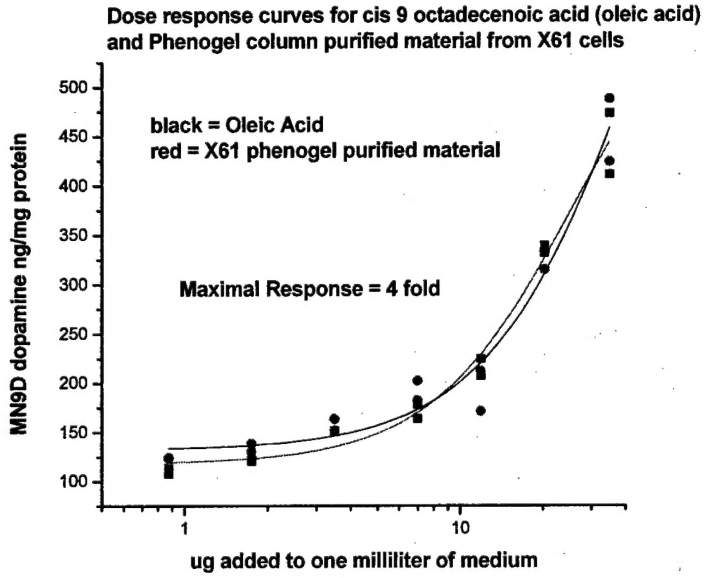


Fig. 3 The NMR spectra of peak #49 from the Phenogel column purified material (top) as compared to commercial oleic acid (bottom). The ^{1}H spectrum is shown at the bottom of each panel, and the peak integral values of the intensities are indicated below the axes. The carboxylic acid and alkene regions of the ^{1}H spectra are magnified and expanded. The alkene regions of the ^{13}C spectra are shown as insets. It is apparent that the Phenogel #49 sample contains one major and one minor species. The minor species was identified by NMR analysis to be cis-vaccenic acid.

A variety of compounds structurally related to oleic acid were obtained and examined by NMR to determine the identity of the minor compound in the sample. We found that the ^{1}H and ^{13}C NMR spectra of cis-vaccenic acid (cis-11-octadecenoic acid), which has the same empirical formula as oleic acid, were essentially identical to those of the minor species in the sample. Thus, our NMR data strongly support the conclusion that the sample comprises a mixture of approximately 80-90% cis-oleic acid and 10-20% cis-vaccenic acid.

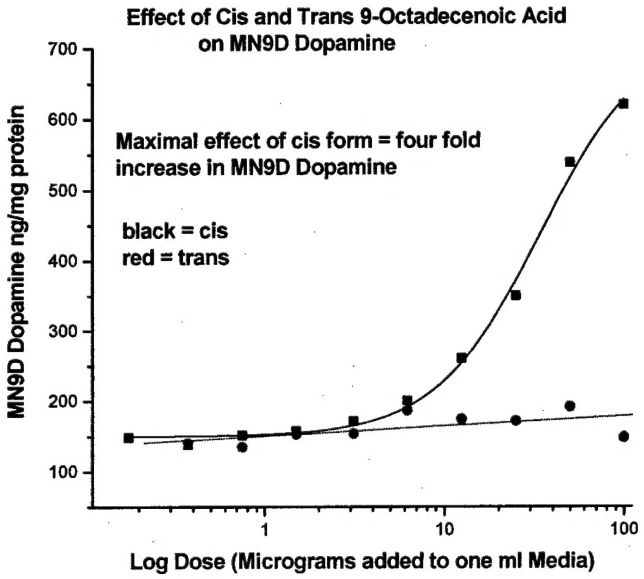
As described above, the spectral analysis with known synthetic entities unequivocally established the chemical composition of the majority of the purified material to be cis-9-octadecenoic acid (oleic acid). The Phenogel purified material and synthetic oleic acid showed identical concentration response in terms of increasing MN9D dopamine as shown in Figure 4.

Figure 4



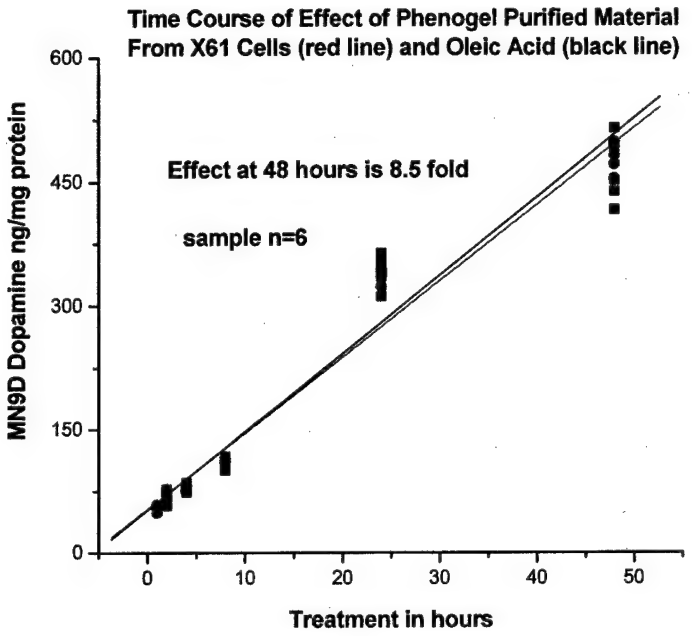
As can be seen in Figure 5, the trans form of oleic acid (elaidic acid) is inactive with respect to a dopaminergic stimulatory effect.

Figure 5



A number of studies have been initiated on the action of the active compound. The first of these studies on the time course of the Phenogel purified material and oleic acid has been completed. It is clear that the full effect of the Phenogel purified material and oleic acid on the MN9D cells requires 48 hours as shown in Figure 6. The maximal effect of the known compound is impressive, resulting, after 48 hours, in an 8.5-fold increase in MN9D dopamine (Figure 6). This experiment was conducted using MN9D cells of low passage and lower basal dopamine concentrations than in previous experiments. Given that the MN9D cells are doubling every 24 hours, this result would suggest that the effect of the active compound is to induce a biochemical alteration in MN9D cells possibly secondary to stimulation of some particular transcriptional event. If that is the case, then the effect should be present in the daughter cells as they appear. This issue will be directly examined by visualization of the cells with respect to tyrosine hydroxylase protein induction by the use of immunocytochemical staining, a routine procedure in our laboratory.

Figure 6



It should be noted that several other fractions from the Superdex and Phenogel columns are capable of increasing MN9D dopamine. In addition, 10% of the material present in the cis-9-octadecenoic acid fraction from the Phenogel column is another chemical entity. Given these findings, the possibility exists that we have found a family of long-chain fatty acid signaling molecules which may be involved in regulation of the dopaminergic phenotype. For this reason, we conducted a limited structure activity study of the effects of a series of long-chain fatty acids on MN9D dopamine content. It is clear from this study, that the ability of oleic acid to increase MN9D dopamine content over a 48 hour period is shared by a number of other long-chain fatty acids containing one to four double bonds, as shown in Table 1. Concentration-response curves were determined for each of the indicated compounds and compared to oleic acid using 7 concentrations over a range of 3 to 124 μM. The majority of the compounds tested were active, but in most cases, a maximal response was not obtained even at 124 μM. For this reason, Table 1 provides the cellular MN9D dopamine level (ng/mg protein) following 48 hours of treatment with 124 μM of each respective compound and the amount of increase this represents over the DMSO vehicle control.

Table 1

Effect of Unsaturated Long-Chain Fatty Acids on MN9D Cellular Dopamine

Compound	Dopamine (ng/mg protein)	Fold-Increase over DMSO Control
Oleic Acid	332	4.8
Arachidonic Acid	365	5.3
Linoleic Acid	300	4.3
Linolenic Acid	297	4.3
Palmitoleic Acid	280	4.0
cis-Vaccenic Acid	264	3.8
cis-13-Octadecenoic Acid	134	1.9
Petroselenic Acid	92	1.3
Oleic Anhydride	81	1.2

MN9D cells were treated for 48 hrs with 124 μM of each of the compounds indicated above and then cellular dopamine content determined. All compounds were dissolved in dimethylsulfoxide (DMSO). Dopamine level of DMSO vehicle control = 69 ng/mg protein.

Two of the compounds tested showed only minimal effects: oleic anhydride, formed by the fusion of two molecules of oleic acid with the splitting out of water, and petroselenic acid, an 18 carbon monoenoic acid in which the double bond is at position 6, in contrast to oleic acid with a double bond at position 9. The remaining seven long-chain fatty acids were all active and included both monoenoic acids (oleic, palmitoleic, cis-vaccenic, and cis-13-octadecenoic acid) and polyenoic acids (linoleic with two double bonds, linolenic with three double bonds and arachidonic with four double bonds).

Thus, it is clear that a variety of long-chain fatty acids are capable of increasing the dopamine content of a mesencephalic-derived immortalized monoclonal cell line expressing a dopaminergic phenotype. While the number and variety of fatty acids tested is too limited to reach systematic conclusions regarding the optimal structure necessary to produce an increase in MN9D dopamine, the ability to significantly elevate MN9D dopamine content appears to depend on the presence of a carboxylic acid group and the relative position of an unsaturated double bond. Both petroselenic and oleic acid are C18 fatty acids. Petroselenic acid, which shows minimal effects (see Table 1), has a cis double bond that is located three carbons further from the terminal carboxylic acid than that of oleic acid, thus suggesting that the length of the side chain may be a critical determinant of effect.

Given the interesting structure activity data obtained to date, we are initiating a collaborative effort with Dr. Viresh Rawal, Professor in the Department of Chemistry at the University of Chicago for the systematic synthesis and testing of fatty acid derivatives with shorter carbon side chains than that present in oleic acid and not present in nature to determine the minimal chemical structures necessary to elicit a dopaminergic stimulatory effect on the MN9D cell.

Probably the most critical initial question is whether the active chemical has a similar effect on dopamine level and dopamine cell survival on primary dopaminergic neurons as we had previously demonstrated for presumptive active peptides [5, 11]. In that regard, a series of studies using aggregate culture and primary dopaminergic neurons will be initiated within the next month in order to provide direct evidence on this point.

Mechanism of action underlying the dopaminergic stimulatory effect of oleic acid

We have now initiated a series of studies on the mechanism of action of oleic acid on MN9D dopamine. While we know that oleic acid increases MN9D dopamine, the mechanism of increase could involve a number of processes. MN9D dopamine could increase due to synthesis, or reduction in metabolism, or by an increase in the capacity of the cell to retain or "store" the dopamine produced. As a first step to assessing the role of synthesis, storage and metabolism in the increase of MN9D dopamine by oleic acid, we have conducted an experiment in which the total amounts of the dopamine, its precursor dihydroxyphenylalanine (DOPA) and its metabolites dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA) in the cells and media were determined in treated and untreated cells. Dopamine is synthesized within the MN9D cell. The amount of DOPA present is a measure of conversion of tyrosine, by tyrosine hydroxylase, and dopamine a measure of conversion of DOPA to dopamine by DOPA decarboxylase. The total amount of dopamine and metabolites in the cells and the media therefore provides a measure of total synthesis. A comparison of cellular to media levels of dopamine permits an assessment of the MN9D cell capacity to retain or store the dopamine it has synthesized. The amounts of the principal metabolites of dopamine, DOPAC and HVA provides an assessment of possible effects of oleic acid on metabolism.

The results of this experiment are given below.

Table 2

Control

	DOPA	Dopamine	DOPAC	HVA
Cellular	0.10	0.23	0.02	ND
Media	1.59	0.99	1.01	3.29

Following 48 hr treatment with 124 uM Oleic acid

Cellular	0.02	1.15	0.27	ND
Media	2.15	1.97	1.28	3.46

Levels are expressed as average total nanomoles (n = 6 cultures/treatment).

While the results are open to a number of interpretations, several things are clear from this experiment. First, it is apparent that in the presence of oleic acid there is an increase in the total amount of dopamine in the culture well. In the case of the untreated control, the total dopamine (media and cells) is 1.22 nanomoles, while in the case of the oleic-treated cultures there are 3.12 nanomoles (a difference of 2.6-fold). The amount of metabolites in the two cases is quite similar. Thus, it would appear that oleic acid increases total dopamine of the MN9D cell through an increase in synthesis rather than a reduction in metabolism. Whether the effect is on the tyrosine hydroxylase or DOPA decarboxylase step will require direct measurements of the amounts or activities of these enzymes.

The data is also suggestive of an increase in uptake and/or storage capacity in the cells treated with oleic acid. Since dopamine is synthesized within the cells and substantial amounts of dopamine in both cases are present in the media, it is probable that the cell has a finite uptake and/or storage capacity (control: 19% of the total dopamine is in the cell; oleic-treated: 36% of the total dopamine is in the cell). The control cells can only retain 0.23 nanomoles of dopamine, but the oleic-treated cells retain 1.15 nanomoles. This could reflect an increase in uptake and/or storage and that interpretation of the data is consistent with our earlier findings that X61 whole lysate from which the oleic acid was purified is apparently capable of increasing MN9D dopamine uptake or storage (see annual report of October 2002).

These findings would suggest that oleic acid increases both synthesis and uptake and/or storage of dopamine by the MN9D cell. However, the data should be interpreted with considerable caution given the possibility of differences in differentiation and compartmentalization states under the two conditions, but the results certainly point to the necessity of measuring MN9D dopamine synthesis and uptake and/or storage and those experiments are in progress.

RESEARCH ACCOMPLISHMENTS

- Further purification using reverse phase chromatography of a highly hydrophilic small peptide in the range of 0.4 to 0.8 kDa obtained from the lysate of X61 cells capable of increasing cellular dopamine. The current state of purification should permit structural analysis of the components of the molecular entities responsible for the activity by tandem mass spectroscopic, fragmentation and nuclear magnetic resonance analysis.

- Purification of an isoamyl alcohol/chloroform extract of a concentrate of the lysate of X61cells, a immortalized monoclonal cell derived from the corpus striatum capable of markedly increasing the dopaminergic content of the dopaminergic MN9D cell. NMR analysis established that the chemical moieties responsible for the activity contained in the isoamyl alcohol/chloroform extract are a mixture of the long chained fatty acids consisting of 90% oleic and 10% vaccinic acid.

- Demonstration that the ability to increase MN9D dopamine by oleic and cis-vaccenic acids is shared by a number of other long-chain fatty acids with one to four double bonds and including arachidonic, linoleic, linolenic, palmitoleic, and cis-13-octadecenoic acid.

- Neurochemical experimental evidence that the elevation of MN9D dopamine by oleic acid involves both an increase in synthesis and cellular uptake and/or storage.

REPORTABLE OUTCOMES

Published Manuscripts (included in the Appendix)

Won, L., Bubula, N., Hessefort, S., Gross, M. and Heller, A. Enhanced survival of primary murine dopaminergic neurons induced by a partially purified cell lysate fraction from striatal derived hybrid monoclonal cells. Neuroscience Letters 353, 83-86, 2003.

Heller, A., Won, L., Bubula, N., Hessefort, S., Kurutz, J.W., Reddy, G.A., and Gross, M. Long-chain fatty acids increase cellular dopamine in an immortalized cell line (MN9D) derived from mouse mesencephalon, Neuroscience Letters, in press.

Patent Application

Provisional Patent Application Entitled: OLEIC ACID AS A THERAPEUTIC FOR TREATING NEUROLOGICAL DISEASES BY by Alfred Heller, Martin Gross and Lisa Won. Reference ARCD: 411USP1 filed on August 23, 2004.

CONCLUSIONS

In conclusion, during the current year, as detailed in the Research Accomplishments section, we have reported a number of new findings which are critical to the primary objectives of the program.

First, it has been possible to produce sufficient purification of a small, hydrophilic peptide fraction obtained from the lysate of the X61 cell to provide sufficient material which should be amenable to structural analysis by tandem mass spectroscopy and fragmentation as well as nuclear magnetic resonance analysis. The purified material from which the hydrophilic peptide fraction was obtained is capable of increasing dopamine in clonal dopaminergic MN9D cells as well as in primary dopaminergic neurons. In addition, this activity is capable of preventing dopaminergic cell death in the absence of appropriate target cells. The activity is of interest from two perspectives. If the molecular structure of this activity could be deduced, given its size, it would represent a unique dopaminergic regulatory moiety of obvious scientific interest. In addition, given that Parkinson's disease is a degenerative disorder involving loss of the dopaminergic neurons of the nigrostriatal projection, considerable interest has focused on the possible therapeutic role of trophic agents. Our discovery of low molecular weight polypeptide fractions, with stimulatory dopaminergic activities, and the ability to prevent dopaminergic cell loss in the absence of target cells may represent a new approach to therapy of the disease.

The primary focus and most successful outcome of the research conducted during the current year has been the purification, isolation and identification of the dopaminergic stimulatory activity present in an isoamyl alcohol/chloroform extract of a lysate concentrate from the X61 cell. The activity is primarily due to the presence of oleic acid in the organic soluble fraction which is capable of causing marked increases in the MN9D dopamine. The mechanism of action of oleic acid may involve the induction of an increased dopaminergic phenotype in these dividing MN9D cells. This discovery has led to the identification of a series of fatty acids with one to four double bonds with similar activity. The mechanism of action of dopamine elevation by oleic acid appears to involve an increase in dopamine synthesis and uptake and/or storage. This finding suggests a novel function for long-chain fatty acids in the regulation of the dopaminergic phenotype. The discovery of this action of the fatty acids is of clear interest with respect to the treatment of Parkinson's disease which arises due to a loss of dopaminergic innervation to the striatum and results in debilitating motor dysfunctions. It is intriguing that a long-chain fatty acid such as oleic acid, which is essentially a benign dietary material, can markedly increase the dopamine content of a cell expressing a dopaminergic phenotype. If it should prove to be the case, that oleic acid or other active fatty acids increase the dopamine content of primary dopaminergic neurons, as we previously reported for the crude X61 lysate and partially purified ultrafiltrates (UF4), the long-chain fatty acids may provide an interesting addition to pharmacological approaches to the investigation of the disease and its treatment.

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Enhanced survival of primary murine dopaminergic neurons induced by a partially purified cell lysate fraction from mouse-derived striatal hybrid monoclonal cells

Lisa Won[a], Nancy Bubula[a], Suzanne Hessefort[a], Martin Gross[b], Alfred Heller[a,*]

[a]*Department of Neurobiology, Pharmacology and Physiology, The University of Chicago, 947 East 58th Street, Chicago, IL 60637, USA*
[b]*Department of Pathology, The University of Chicago, Chicago, IL 60637, USA*

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Abstract

Lysates of X61, a striatal-derived cell line, and a partially purified preparation from the lysate (UF4) contain a factor(s) capable of increasing the dopamine content of a mesencephalic-derived dopaminergic cell line (MN9D) and of cultures containing primary dopaminergic neurons. Treatment of cultures containing dopaminergic primary neurons grown in the absence of target cells over a 2 week period with X61 lysate or UF4 resulted in an elevation of dopamine levels of the cultures and of media homovanillic acid as well as a 2.0-fold (UF4) to 2.9-fold (X61 lysate) increase in the density of dopaminergic neurons in treated cultures. The results suggest that the activity factor derived from X61 is capable of preventing dopaminergic cell loss which occurs in the absence of dopaminergic target cells of the corpus striatum.

Keywords: Dopamine; Nigrostriatal; Trophic factor; Parkinson's disease; Cell lines; MN9D; Reaggregate culture

We have previously reported that the lysate of an immortalized, monoclonal cell line (X61) derived from the striatum is capable of increasing the dopamine (DA) content of a mesencephalic monoclonal hybrid cell line derived from the mesencephalon expressing a dopaminergic phenotype (MN9D) [3]. This lysate also increases the DA content of three-dimensional reaggregate cultures containing primary dopaminergic neurons [3]. The reaggregate cultures consist of fetal mesencephalon co-cultured with optic tectum, an area of brain which does not receive a dopaminergic innervation. Under these circumstances, fewer dopaminergic neurons are observed, possibly secondary to cell death, as compared to the quantitative survival of such cells in reaggregate cultures containing dopaminergic striatal target cells [2]. The present experiment was conducted to determine whether the rise in DA levels in the reaggregate cultures seen with exposure to X61 cell lysate or with a partially purified, ultrafiltrate fraction of the lysate (UF4) [4] was associated with an increase in the survival of dopaminergic neurons in the absence of target cells.

Reaggregate cultures were prepared from embryonic day 14 C57BL/6 mouse brains by dissecting the mesencephalic tegmentum (containing developing dopaminergic neurons) and the tectum (a region to which dopaminergic neurons do not project) (see Ref. [6] for method details). The tissues were dissociated into single cell suspensions and counted using a hemacytometer. A total of 3.25 million mesencephalic cells were dispensed along with 6.5 million tectal cells into 25 ml Erlenmeyer flasks containing 3.5 ml of initial culture medium composed of Basal Medium Eagle's, 10% (v/v) fetal bovine serum, 1% (v/v) penicillin/streptomycin (5000 units penicillin/5000 μg streptomycin) and 0.025% (w/v) deoxyribonuclease I. The flasks were prepared and placed into a rotatory incubator where reaggregates formed over the course of 24 h. After the first 24 h, the medium was removed and replaced with fresh medium containing horse serum instead of fetal bovine serum. At this time, the reaggregates were treated with X61 cell lysate (12.5 μl/ml; $n = 5$ flasks), UF4 (20 μl/ml; $n = 6$ flasks) or with appropriate volumes of phosphate buffered

* Corresponding author. Tel.: +1-773-702-3513; fax: +1-773-702-3774.
E-mail address: effe@midway.uchicago.edu (A. Heller).

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saline (PBS) vehicle (12.5 μl/ml for X61 cell lysate ($n = 5$) or 20 μl/ml for UF4 ($n = 6$) treatment). The culture media were changed every 2 days and fresh amounts of X61 cell lysate, UF4 or PBS were added. After 8 days in culture, reaggregates from the flasks for a given treatment group (X61, UF4 or PBS) were combined and redistributed to five or six experimental flasks. The pooling of reaggregates and their re-distribution into experimental flasks reduces the variance among flasks subjected to the same experimental treatment [6]. The cultures were maintained in this manner for 15 days at which time reaggregates and media were collected and biochemical and histological examinations were conducted. For this experiment, culture media were prepared using sera which had been previously dialyzed to remove endogenous serotonin [6].

X61 cell lysate was prepared by sonicating X61 cells in PBS at room temperature. Purification of the active material from the lysate was facilitated by the finding that the dopaminergic stimulatory activity can be largely converted from a high molecular weight protein fraction to a much lower molecular weight protein fraction when the lysate is allowed to incubate at room temperature for up to 2 days. Amicon YM-5 membranes (5000 Da mol. wt. cut-off) were utilized to separate a low molecular weight stimulatory fraction (UF4) from the X61 lysate. After sonication, the lysate was concentrated by pressure filtration and allowed to 'autodigest' at room temperature overnight. The next morning, the lysate was diluted with PBS to its initial volume and re-concentrated by pressure filtration. After an additional overnight 'autodigestion' and re-dilution on day 2, the lysate was re-concentrated once more, generating the UF4 ultrafiltrate fraction. The conversion of the activity from high to lower molecular weight fractions, in all likelihood, represents an enzymatic breakdown of higher molecular weight material. Active lower molecular weight fractions are stable at room temperature despite the finding that the crude cell lysate activity is labile to boiling [3]. The heat lability may represent a precipitation or coagulation of protein entrapping the activity. Dopaminergic stimulatory activity (i.e. the ability to increase cellular DA content) of the ultrafiltrates obtained from each daily filtration was assessed as previously described [3] using a mesencephalic-derived, dopaminergic cell line, MN9D. Of the ultrafiltrates obtained from each daily filtration, the UF4 ultrafiltrate (following 48 h incubation at room temperature) was the most stimulatory and, therefore, was the preparation used to treat the reaggregate cultures described below. The stimulatory activity in UF4 is approximately equal to that of the initial X61 lysate, and UF4 is approximately 60-fold purer based on absorbance at 230 nm.

Samples of culture media and reaggregates were collected at 15 days from each flask for HPLC analysis of DA and homovanillic acid (HVA) levels. The protein content of the reaggregates was determined spectrophotometrically [12].

The density of dopaminergic cells in the reaggregates was visualized using immunocytochemical methods and estimated based on the procedure previously described by Heller et al. [6] and validated by computer simulation methods [8]. The cultures were fixed with 4% paraformaldehyde, embedded in gelatin and sectioned (50 μm) with a vibratome. Tyrosine hydroxylase (TH) immunocytochemistry was performed on free-floating tissue sections using standard peroxidase anti-peroxidase techniques. Briefly, the number of TH-positive cells from a given flask was estimated by counting the cells from a random selection of 30 sections. A digital image was captured of each of the 30 reaggregate sections used for counting with a Nikon Coolpix 995 digital camera. The area (in pixels) of each reaggregate section was obtained using Adobe Photoshop 7.0. The pixel area from each reaggregate section was converted to square microns and multiplied by 50 (section thickness) to obtain the sectional volume. The density of TH cells was obtained by dividing the number of TH cells counted by the sum of sectional volumes from the 30 reaggregate sections.

In the case of reaggregates treated with X61 cell lysate or the UF4 ultrafiltrate, it was apparent from gross inspection that many of the reaggregate sections contained considerably more TH-positive neurons than the corresponding PBS controls (shown for UF4 treatment in Fig. 1). The results of the quantitative cell counting and neurochemical analysis of reaggregate tissue and media are presented in Table 1.

It is clear from this study that the X61 cell lysate as well as the UF4 ultrafiltrate are capable of preventing a loss of dopaminergic neurons in the absence of dopaminergic target cells in reaggregate culture. The neurochemical changes observed most probably are a result of the increased density of neurons expressing a dopaminergic phenotype in the treated reaggregate cultures. While DA is only increased by 25–30%, this may be a reflection of the immaturity of the neuronal population of the reaggregates. In addition, since no target cells were present in mesencephalic-tectal reaggregates of the current study, axonal process formation

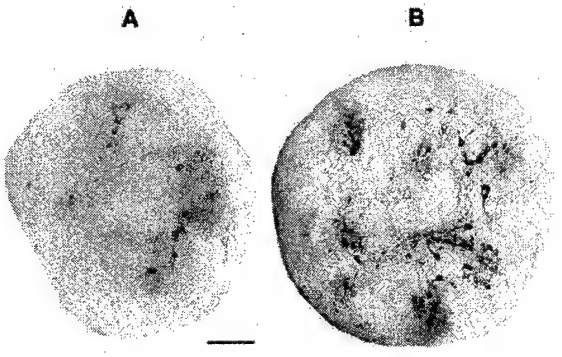

Fig. 1. Sections of mesencephalic-tectal reaggregates with dopaminergic neurons visualized by tyrosine hydroxylase (TH) immunocytochemistry. Microscopic examination of section A (PBS-treated reaggregate) showed that it contained 11 TH labeled cells while section B (UF4-treated reaggregate) contained 35 such cells. Scale bar: 100 μm.

Table 1
Effect of X61-derived dopaminergic stimulatory factor(s) on survival of dopaminergic neurons

	TH cell density	Reaggregate DA	Media HVA
Ultrafiltrate treatment			
UF4	563 ± 131/mm^3*	10.4 ± 0.02***	92.9 ± 1.0***
PBS	196 ± 48/mm^3	7.9 ± 0.22	53.1 ± 0.1
Cell lysate treatment			
X61 cell lysate	839 ± 85/mm^3**	8.5 ± 0.1***	185.0 ± 20.3***
PBS	415 ± 99/mm^3	6.4 ± 0.3	47.5 ± 1.7

Values given are the mean ± SEM of $n = 5$ for the UF4/PBS and $n = 6$ for X61 cell lysate/PBS treatment. Reaggregate dopamine (DA) is expressed as ng/mg protein. Media homovanillic acid (HVA) is expressed as ng/ml of media/mg reaggregate protein. Significantly different than PBS control: *$P < 0.025$, **$P < 0.01$, ***$P < 0.001$.

is at best minimal, limiting DA storage [10]. The dopaminergic neurons within the mesencephalic-tectal reaggregates are, however, clearly neurochemically active and release DA as evidenced by the 1.8- (treatment with UF4) to 3.9-fold (treatment with X61 cell lysate) increase in media content of the DA metabolite, HVA, which provides an estimate of released transmitter (Table 1).

Anatomic loss of dopaminergic neurons of the nigrostriatal projection is a central feature of Parkinson's disease (for review see Ref. [9]). It is clear that one cause of such degeneration is a loss of contact of the mesencephalic dopaminergic neurons with their striatal targets as evidenced by the fact that lesions which transect the projection of dopaminergic neurons to the striatum result in a loss of cellular components of the substantia nigra [11].

It is a reasonable assumption that in the absence of striatal target cells (mesencephalic-tectal reaggregates) there is an actual loss of the dopaminergic neurons as occurs in vivo following various insults, but the possibility that the cells are present, but no longer express a dopaminergic phenotype cannot be excluded. In either case, there are clearly fewer neurons expressing a dopaminergic phenotype in mesencephalic-tectal as compared to mesencephalic-striatal cultures. The three-dimensional reaggregate system, therefore, provides a reasonable model system for the examination of agents capable of preventing dopaminergic cell loss following either loss or separation from dopaminergic target cells.

X61 cell lysate or partially purified fractions from this material (i.e. UF4 ultrafiltrate) contain an activity capable of increasing the DA content of dopaminergic MN9D monoclonal hybrid cells or of three-dimensional reaggregates containing primary dopaminergic neurons in the absence of targets [3]. As demonstrated here, it would appear that this latter effect represents an actual increase (2.0- to 2.9-fold) in the density of dopaminergic neurons surviving in the cultures. The results obtained with the X61-derived dopaminergic stimulatory factor(s) are quite similar to a previous experiment in which we demonstrated that the addition of fetal striatal membrane preparations to mesencephalic-tectal reaggregates resulted in an increased survival of dopaminergic neurons, in some cases almost to the extent of the survival achieved with striatal cells [5]. The result suggested that 'dopaminergic survival factors' associated with membranes were present in the striatum. We did not pursue this finding since the reaggregate system was much too lengthy an assay procedure for the purposes of purification of the cellular-derived activity. Instead, we turned our attention to the use of somatic cell hybridization methods to produce the current monoclonal hybrid dopaminergic and striatal cells [1,7,13]. The basic hypothesis was that the monoclonal striatal cells, such as X61, would produce dopaminergic 'trophic' factors, and the monoclonal dopaminergic cells, MN9D, would serve as a test object for such factors. The data provided here would appear to justify that approach.

The cell-saving activity present in X61 cell lysate is obviously of considerable interest. We have previously demonstrated that the X61 lysate does not contain a significant number of the known trophic factors for dopaminergic neurons [3]. The UF4 ultrafiltrate factor(s) represents dopaminergic stimulatory activity which is less than 5000 Da. The activity in UF4, capable of increasing MN9D DA content, has now been purified some 50,000-fold by gel filtration and charcoal treatment (unpublished observations) and will be tested with regard to its ability to prevent dopaminergic cell loss in the absence of target cells in three-dimensional reaggregate culture as seen with ultrafiltrate (UF4) preparations.

The exact chemical nature and mechanism of action of the X61 cell lysate activity is still under investigation. Demonstration and concentration of the activity was made possible by the availability of an appropriate test object, the dopaminergic MN9D cell. The effect of the lysate activity on such monoclonal cells is to increase DA content. As demonstrated here, the biological effect of the partially purified lysate activity includes the ability to prevent the loss of primary dopaminergic neurons which occurs in the absence of target cells. The lysate activity has obvious utility in the investigation and treatment of Parkinson's disease in terms of elevation of the transmitter level in surviving cells, prevention of progressive cell loss and as an adjunct to dopaminergic cell survival in fetal transplantation.

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Long-chain fatty acids increase cellular dopamine in an immortalized cell line (MN9D) derived from mouse mesencephalon

Alfred Heller[a,*], Lisa Won[a], Nancy Bubula[a], Suzanne Hessefort[a], Josh W. Kurutz[b], Giridher A. Reddy[c], Martin Gross[d]

[a] *Department of Neurobiology, Pharmacology and Physiology, The University of Chicago, 947 East 58th Street, Chicago, IL 60637, USA*
[b] *Department of Biochemistry and Molecular Biology, The University of Chicago, Chicago, IL 60637, USA*
[c] *Department of Pediatrics, The University of Chicago, Chicago, IL 60637, USA*
[d] *Department of Pathology, The University of Chicago, Chicago, IL 60637, USA*

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Abstract

The lysate of an immortalized monoclonal cell line derived from the striatum (X61) contains a dopaminergic stimulatory activity that is capable of increasing the dopamine content of an immortalized mouse mesencephalic cell line (MN9D) which expresses a dopaminergic phenotype. Purification of an isoamyl alcohol extract of this lysate and subsequent identification by NMR spectroscopic analysis demonstrated that the dopaminergic stimulatory activity contained within the lysate was a mixture of 80–90% *cis*-9-octadecenoic acid (oleic acid) and 10–20% *cis*-11-octadecenoic acid (*cis*-vaccenic acid). The effect of oleic acid on MN9D dopamine is a prolonged event. MN9D dopamine increases linearly over a 48 h period suggesting the induction of an increased dopaminergic phenotype in these dividing cells. The ability to increase MN9D dopamine by oleic and *cis*-vaccenic acids is shared by a number of other long-chain fatty acids including arachidonic, linoleic, linolenic, palmitoleic, and *cis*-13-octadecenoic acid. The possibility that oleic or other relatively innocuous fatty acids might affect dopaminergic function in primary neurons is intriguing with respect to possible therapeutic approaches to the treatment of dopaminergic cell loss and the motor sequelae of Parkinson's disease.

Keywords: Dopamine; Fatty acids; MN9D; Nigrostriatal; Parkinson's disease; Trophic factor

Immortalized monoclonal cells of the mouse nigrostriatal projection have been developed as an approach to the identification of substances which could regulate dopaminergic function and cell survival [1,14]. The dopaminergic MN9D cell line of mesencephalic origin and the X61 cell line of striatal origin were obtained by somatic cell fusion with the N18TG2 neuroblastoma which is lacking the hypoxanthine phosphoribosyltransferase enzyme [1,14]. We have previously demonstrated that striatal-derived monoclonal cells (X61) contain dopaminergic stimulatory substances which increase the dopamine content of MN9D cells [3]. Striatal cell lines (X61) provide a source for such substances and the mesencephalic-derived MN9D cell line provides a rapid assay method for detecting active molecules capable of modulating cellular dopamine. The crude cell lysate of X61 cells, as well as a partially purified ultrafiltrate preparation (UF4) of that lysate, also increases the dopamine content of primary dopaminergic neurons grown in reaggregate culture in the absence of target cells (i.e., mesencephalic cells co-cultured with tectum, a non-target region for dopaminergic neurons) as well as levels of homovanillic acid in the culture medium [15]. In such cultures, in which the majority of dopaminergic neurons are lost due to the absence of target cells, treatment with the crude lysate or UF4 ultrafiltrate results in a 2- (UF4) to 2.9- (X61 lysate) fold increase in the density of dopaminergic neurons in the treated cultures [15].

The UF4 ultrafiltrate contains active substances, probably peptides, of low molecular weight and high water solubility. It was, however, apparent that the bulk (two-thirds) of

* Corresponding author. Tel.: +1 773 702 3513; fax: +1 773 702 3774.
E-mail address: effe@midway.uchicago.edu (A. Heller).

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dopaminergic stimulatory activity present in the processed X61 cells resided in a fraction which did not pass through a YM-5 ultrafiltration membrane and was lipid soluble. The present study was conducted to determine the chemical nature and activity of this major fraction.

The UF4 ultrafiltrate was obtained from sonicated lysates of X61 cells which were allowed to "autodigest" at room temperature for 2 days and concentrated by pressure filtration through an Amicon YM-5 membrane (5000 Da molecular weight cut-off). Some form of digestion occurs in this process as evidenced by the fact that 2 days of incubation at room temperature results in the conversion of some (approximately 30%) of the dopaminergic stimulatory activity in the X61 cell lysate from a high molecular weight form to a size that can pass through a YM-5 membrane (see [15] for details).

The low molecular weight UF4 ultrafiltrate fraction contained significant dopaminergic stimulatory activity as assessed by effects on MN9D cells. However, the majority of the activity from the "autodigested" X61 cell lysate did not pass through the YM-5 ultrafiltration membrane. Approximately two-thirds of the activity resided in the material remaining on the high molecular weight side of the Amicon YM-5 membrane and is referred to as "X61 concentrate". This X61 concentrate was subsequently extracted with 2 M NaCl followed by a 1:1 mixture of isoamyl alcohol/chloroform. The isoamyl alcohol/chloroform extract was shown to contain materials capable of increasing MN9D dopamine levels. This activity is not extractable from fresh X61 cell lysate, but appears to require the autodigestion step with time for the activity to become liberated from some cell component and be available for organic extraction.

The isoamyl alcohol/chloroform soluble stimulatory activity was taken up by a C18 reverse phase column from a mixture of 70% acetonitrile/30% (0.05% trifluoroacetic acid in water) and then eluted by a linear gradient from the mixture to 100% acetonitrile. The active fractions from the column showed some absorbance at 215 nm, but the bulk of absorbance was seen in fractions devoid of activity, suggesting that the reverse phase separation resulted in considerable purification of the activity. The active fractions from the reverse phase column were then applied to a Phenomenex 5 μm, 50 Å Phenogel gel filtration sizing column. The dopaminergic stimulatory activity eluted from the Phenogel column in 100% acetonitrile within a single absorbance peak. The Phenogel fractions containing dopaminergic stimulatory activity were subjected to mass spectrographic analysis. Two peaks of high intensity were observed with molecular weights of 283 and 565.

NMR spectroscopy demonstrated that the single elution peak from the Phenogel column contained two chemical moieties, the greater of which constituted approximately 80–90% of the material (Fig. 1). The 600 MHz ^{1}H 1D NMR spectrum of the sample showed one major set of peaks and no significant minor peaks, suggesting a sample purity greater than 95%. The ^{13}C 1D NMR spectrum, however, showed two sets of peaks, indicating that the sample contained two species

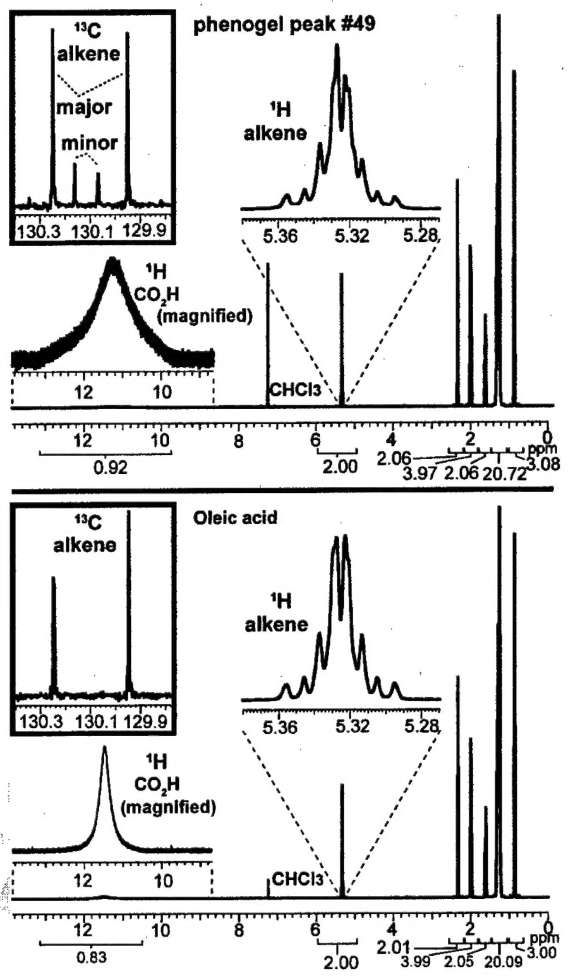

Fig. 1. The NMR spectra of peak #49 from the Phenogel column purified material (top) as compared to commercial oleic acid (bottom). The ^{1}H spectrum is shown at the bottom of each panel, and the peak integral values of the intensities are indicated below the axes. The carboxylic acid and alkene regions of the ^{1}H spectra are magnified and expanded. The alkene regions of the ^{13}C spectra are shown as insets. It is apparent that the Phenogel #49 sample contains one major and one minor species. The minor species was identified by NMR analysis to be *cis*-vaccenic acid.

so closely related that they gave rise to virtually identical ^{1}H NMR spectra. The minor component made up approximately 10–20% of the total sample, according to their relative intensities in the ^{13}C spectra. The positions of the ^{1}H and ^{13}C peaks in these spectra ruled out the possibility that the sample contains protein, peptide, DNA, RNA, carbohydrate, glycolipid, steroid, or other cholesterol-related molecules. The positions are instead consistent with those expected from a fatty acid. Comparison of the major species' spectra with ^{1}H and ^{13}C NMR spectra of various fatty acids showed that they were very similar to those of *cis*-9-octadecenoic acid (oleic acid). It remained uncertain whether the unsaturated bond was of *cis* or *trans* configuration. Pure *cis*-9-octadecenoic acid and *trans*-9-octadecenoic acid (elaidic acid) (Aldrich), and their ^{1}H and ^{13}C NMR spectra were compared to those of the sam-

ple. We found that the major species' peaks were essentially identical to those of the *cis* isomer (i.e., oleic acid) (Fig. 1).

A variety of compounds structurally related to oleic acid were obtained and examined by NMR spectroscopy to determine the identity of the minor compound in the sample. We found that the ^{1}H and ^{13}C NMR spectra of *cis*-vaccenic acid (*cis*-11-octadecenoic acid), which has the same empirical formula as oleic acid, were essentially identical to those of the minor species in the sample. Thus, our NMR data support the conclusion that the purified sample comprises a mixture of approximately 80–90% *cis*-oleic acid and 10–20% *cis*-vaccenic acid.

The NMR spectral analysis with known synthetic entities established the chemical composition of the majority of the purified material to be *cis*-9-octadecenoic acid (oleic acid). The mass spectroscopic analysis is in accord with the NMR data in that the molecular weight of 283 corresponds to that of oleic acid and/or *cis*-vaccenic acid and the larger sized 565 molecular weight species may well represent a dimerization of these long-chain fatty acids.

In order to determine whether the Phenogel purified material and synthetic oleic acid produce similar effects on MN9D cellular dopamine, MN9D cells were plated into six-well culture plates and cultured in Dulbecco's Modified Eagle's medium containing 5% (v/v) Fetal Clone III and 1% (v/v) penicillin–streptomycin (5000 units penicillin/5000 μg streptomycin). The cells were exposed to increasing concentrations of oleic acid or the Phenogel purified material, diluted in dimethylsulfoxide (DMSO), for 48 h and then collected for analysis of cellular dopamine content using high performance liquid chromatography. Protein content of the cultures was determined spectrophotometrically [11].

In agreement with the NMR data, the Phenogel purified material and synthetic oleic acid showed identical concentration–response in terms of increasing MN9D dopamine as shown in Fig. 2. The concentration–response of MN9D cells to oleic acid has been repeated in two other experiments and the results obtained were identical. Exposure of MN9D cells to concentrations of oleic acid or the Phenogel purified material greater than 124 μM produced either less of a dopaminergic stimulatory effect or was actually toxic to the cells. Thus, the response at 124 μM was considered to be maximal. The maximal effect of oleic acid and the Phenogel purified material in this experiment represents an approximate five-fold increase in dopamine level over controls. The EC_{50} for oleic acid is approximately 5.5×10^{-5} M. The effects of oleic acid are not secondary to either an increase in MN9D cell proliferation or differentiation. This issue was tested directly in a separate experiment in which dopamine levels, cell number and the state of cell differentiation were examined. MN9D cells were exposed to DMSO or 124 μM oleic acid for 48 h. An increase in dopamine of 8.3-fold was observed in this experiment. The protein content of MN9D cells exposed to oleic acid was essentially identical to that of the DMSO group and the number of cells in the oleic acid-treated group (0.89 ± 0.13 million cells, mean ± S.E.M., $n = 6$) was not significantly different from DMSO vehicle (1.05 ± 0.10 million cells, mean ± S.E.M., $n = 6$). The oleic acid-exposed cells showed none of the characteristics of differentiated MN9D cells, i.e. a reduction in cell number or increased process outgrowth [1].

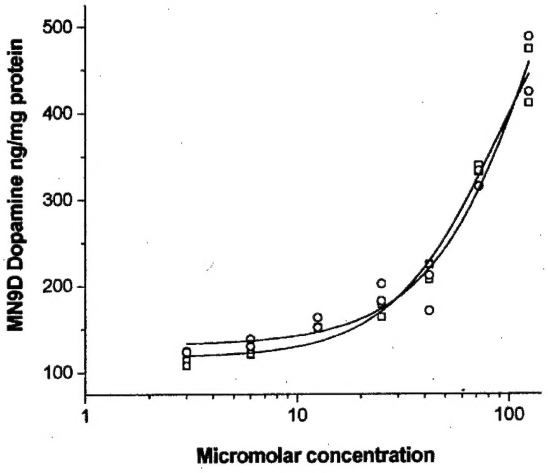

Fig. 2. Effect of 48 h exposure to increasing (log scale) concentrations (3–124 μM) of *cis*-9-octadecenoic acid (oleic acid, squares) or to the Phenogel column purified material from X61 cells (circles) on MN9D dopamine levels. MN9D dopamine level of DMSO vehicle control = 109.9 ng/mg protein.

Given that the assays were carried out in serum-containing medium and the known capacity of oleic acid to bind to serum proteins [8,12], the free EC_{50} for oleic may be considerably lower than this estimate. The level of oleic acid required to increase dopamine levels under serum-free conditions cannot be tested with MN9D cells since they do not grow well under conditions of low serum or serum-free medium. The MN9D cells, in addition, only permit the examination of effects on the catecholaminergic phenotype. The issue of whether oleic acid affects other transmitter phenotypes will require primary neuronal cultures.

While *cis*-vaccenic acid, the minor constituent of the Phenogel purified fraction, is active (see below), a comparison of the concentration–response curve of *cis*-vaccenic with oleic acid on MN9D dopamine, suggests that *cis*-vaccenic acid has a slightly lower potency.

A comparison of the effect of oleic versus the *trans* isomer (elaidic acid), using 11 concentrations ranging from 0.3 to 353 μM, on MN9D dopamine revealed that elaidic acid showed only minimal activity (less than 10% elevation in dopamine even at the highest concentration of 353 μM) (data not shown).

A number of more detailed studies have been initiated on the effect of oleic acid in increasing MN9D dopamine. The first of these studies, a time course on the effect of the Phenogel purified material and oleic acid, demonstrates a linear increase in MN9D dopamine over 48 h (Fig. 3). The effect of both the known compound and the Phenogel purified material in this experiment is impressive, resulting, after 48 h, in at

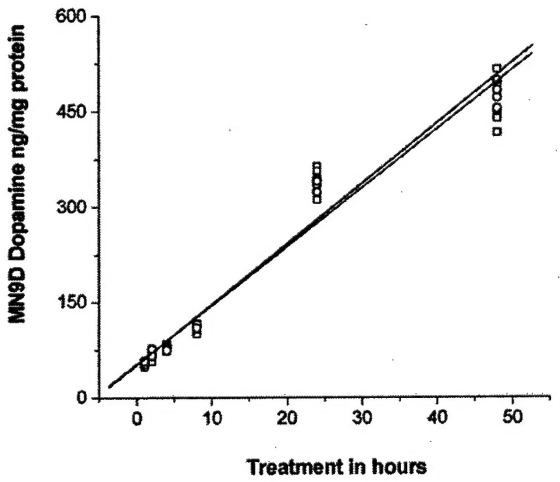

Fig. 3. Time course of the effect of exposing MN9D cells to 124 μM of *cis*-9-octadecenoic acid (oleic acid, squares) or 124 μM of the Phenogel column purified material from X61 cells (circles) on cellular dopamine levels. $N = 6$ cultures per time point. MN9D dopamine level (ng/mg protein) of DMSO vehicle control for the various time points: 1 h, 54.1; 2 h, 63.3; 4 h, 63.6; 8 h, 65.3; 24 h, 77.3; 48 h, 67.5.

least an 8.5-fold increase in MN9D dopamine as was seen in the experiment on cell proliferation described above. Given that the MN9D cells, which are fusion products of mesencephalic cells and the N18TG2 neuroblastoma, are doubling every 24 h, this result would suggest that the effect of the active chemical is to induce an increase in the dopaminergic phenotype of MN9D cells. If that is the case, then the effect should be present in the daughter cells as they appear.

As part of this study, a limited structure–activity analysis was conducted using a variety of common long-chain unsaturated fatty acids. It is clear from this study, that the ability of oleic acid to increase MN9D dopamine content over a 48 h period is shared by a number of other long-chain fatty acids containing one to four double bonds, as shown in Table 1. Concentration–response curves were determined for each of the indicated compounds and compared to oleic acid using seven concentrations over a range of 3–124 μM. The majority of the compounds tested were active, but in most cases, a maximal response was not obtained even at 124 μM. For this reason, Table 1 provides the cellular MN9D dopamine level (ng/mg protein) following 48 h of treatment with 124 μM of each respective compound, and the amount of increase this represents over the DMSO vehicle control.

Table 1
Effect of unsaturated long-chain fatty acids on MN9D cellular dopamine

Compound	Dopamine (ng/mg protein)	Fold-increase over DMSO control
Oleic acid	332	4.8
Arachidonic acid	365	5.3
Linoleic acid	300	4.3
Linolenic acid	297	4.3
Palmitoleic acid	280	4.0
cis-Vaccenic acid	264	3.8
cis-13-Octadecenoic acid	134	1.9
Petroselenic acid	92	1.3
Oleic anhydride	81	1.2

MN9D cells were treated for 48 h with 124 μM of each of the compounds indicated above and then cellular dopamine content determined. All compounds were dissolved in dimethylsulfoxide (DMSO). Dopamine level of DMSO vehicle control = 69 ng/mg protein.

Two of the compounds tested showed only minimal effects: oleic anhydride, formed by the fusion of two molecules of oleic acid with the splitting out of water, and petroselenic acid, an 18 carbon monoenoic acid in which the double bond is at position 6, in contrast to oleic acid with a double bond at position 9. The remaining seven long-chain fatty acids were all active and included both monoenoic acids (oleic, palmitoleic, *cis*-vaccenic, and *cis*-13-octadecenoic acid) and polyenoic acids (linoleic with two double bonds, linolenic with three double bonds and arachidonic with four double bonds).

Thus, it is clear that a variety of long-chain fatty acids are capable of increasing the dopamine content of a mesencephalic-derived immortalized monoclonal cell line expressing a dopaminergic phenotype. While the number and variety of fatty acids tested is too limited to reach systematic conclusions regarding the optimal structure necessary to produce an increase in MN9D dopamine, the ability to significantly elevate MN9D dopamine content appears to depend on the presence of a carboxylic acid group and the relative position of an unsaturated double bond. Both petroselenic and oleic acid are C18 fatty acids. Petroselenic acid, which shows minimal effects (see Table 1), has a *cis* double bond that is located three carbons further from the terminal carboxylic acid than that of oleic acid, thus suggesting that the length of the side chain may be a critical determinant of effect.

The MN9D cell line has served as a useful test object for monitoring the presence and purification of dopaminergic stimulatory activities from lysate of immortalized monoclonal cell lines (X61) derived from the striatum. In addition, at least with respect to the small, water-soluble activity (UF4) which appears to be peptide in nature, effects on the MN9D line were predictive of the ability of this substance to increase the dopamine content of primary dopaminergic neurons and prevent their loss in the absence of striatal targets [15]. Whether the increases in MN9D dopamine seen following treatment with known long-chain fatty acids described here will be replicated on primary dopaminergic neurons is obviously a critical question and is currently being examined using three-dimensional reaggregate culture in a similar manner to the previous studies [3,15].

The striatal lines were developed specifically for the purposes of providing a substantial source of monoclonal cells which could be probed for substances that might influence dopaminergic function, either with respect to dopamine levels, cell survival, or the maintenance of the phenotype. It is worth noting that while an isoamyl alcohol/chloroform extract of a lysate concentrate of the X61 cell yielded a fraction capable of markedly increasing MN9D dopamine, that fraction on purification turned out to contain

long-chain fatty acids (oleic and *cis*-vaccenic acid) which could have been extracted from any number of sources. The presence of these active moieties in the X61 cell line, however, directed our attention to their isolation and purification.

Although this is the first description of an ability of long-chain fatty acids to increase cellular dopamine, there are many reports of effects of oleic and other unsaturated fatty acids on neuronal function. Oleic acid is an activator of protein kinase C activity [4,7] and, as has been reported, its synthesis has been linked to neuronal differentiation during development [6,13] and the promotion of axonal growth and induction of MAP-2 expression (microtubule associated protein-2), a marker of dendritic differentiation [9]. Arachidonic acid markedly stimulates, in a dose-dependent fashion, the spontaneous release of dopamine in purified synaptosomes from rat striatum and inhibits dopamine uptake [5]. In addition, reduced dietary intake of omega-6 (arachidonic acid) and omega-3 (docosahexanoic acid) fatty acids in piglets during the first few weeks of postnatal life has been shown to result in lower brain monoamine concentrations which can be reversed upon supplementation with adequate levels of these fatty acids [2]. We are not aware of any reports of beneficial effects of dietary unsaturated fatty acids on the pathogenesis or clinical course of Parkinson's disease.

While the treatment of the motor sequelae of Parkinson's disease has received intense study for over four decades with some very notable successes [10], it remains clear that additional treatment modalities would be helpful and are being sought. It is intriguing in this regard that a long-chain fatty acid such as oleic acid, which is essentially a benign dietary material, can markedly increase the dopamine content of a cell expressing a dopaminergic phenotype. If it should prove to be the case, that oleic acid or other active fatty acids increase the dopamine content of primary dopaminergic neurons, as we previously reported for the crude X61 lysate and partially purified ultrafiltrates (UF4), the long-chain fatty acids may provide an interesting addition to pharmacological approaches to the investigation of the disease and its treatment.

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